

Molecular cloning of a novel protein-tyrosine phosphatase containing a membrane-binding domain and GLGF repeats

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Abstract

A full-length cDNA encoding a novel cytosolic protein-tyrosine phosphatase (PTP), PTP-BAS, was cloned from human basophils. Due to in-frame deletions in the coding region, PTP-BAS exists in three isoforms: 7,455 bp (2,485 aa) for type 1, 7,398 bp (2,466 aa) for type 2 and 6,882 bp (2,294 aa) for type 3. All three isoforms contain a single PTP catalytic domain at the carboxyl termini as well as two distinct structural sequences. Amino terminal sequences of 300 amino acids are homologous to membrane-binding domains of cytoskeleton-associated proteins. Three 90 amino acid internal repetitive sequences are homologous to the GLGF repeats found in guanylate kinase proteins. PTP-BAS was expressed in various human tissues, especially highly in the kidney and lung. Interestingly, the BAS mRNA level in the fetal brain was remarkably high.

Key words: Protein-tyrosine phosphatase; PTP-BAS; Human basophil; Molecular cloning; Membrane-binding domain; GLGF repeat

Introduction

The cross-linking of high affinity IgE receptors (FcεRI) on basophils and mast cells leads to the immediate release of allergic mediators. Studies using murine cell lines have demonstrated that FcεRI-mediated tyrosine phosphorylation is a major initial signal transducer. Protein-tyrosine kinases (PTKs) of the Src family (Lyn, c-Src and c-Yes [1]) and PTK72 [2] associate with the receptor and become activated after FcεRI cross-linking, leading to tyrosine phosphorylation of multiple proteins. Such enhanced phosphorylation is diminished immediately when cross-linking is reversed [3,4]. Thus, dephosphorylation by protein-tyrosine phosphatases (PTPs) has also been considered to be important in initial signal transduction via FcεRI [5], but this has not been investigated in detail [6,7]. Human basophils and mast cells have not been studied widely, because they are difficult

to obtain and human cell lines have not yet been established.

We have been able to cultivate and differentiate human basophils [8]. In the present study, we isolated them using a fluorescence-activated cell sorter (FACS). PTPs expressed in the isolated basophils were identified by polymerase chain reaction (PCR) using degenerative primers. A novel PTP (named PTP-BAS) was found. Its full-length cDNA was cloned, sequenced and characterized.

2. Materials and methods

2.1. Reagents

Fluorescein amidite (FluorePrime), the mRNA purification Kit, the QuickPrep Micro mRNA Purification Kit and the Ready-To-Go-DNA Labeling Kit were obtained from Pharmacia. The pCR II vector was from a TA Cloning Kit (Invitrogen). The SUPERScript Lambda System, SUPERScript Reverse Transcriptase, 5' RACE System and dsDNA Cycle Sequencing System were purchased from GIBCO BRL. Oligo(dT)15 was from Boehringer Mannheim. The λZAP II/EcoRI Cloning Kit and Gigapack II Gold packaging extract were obtained from Stratagene. Multiple Tissue Northern Blots and a human G3PDH control probe were provided by CLONTECH.

2.2. Basophil-enriched cell culture

KU812E, a subclone of the human chronic myelogenous leukemic cell line, KU812, was obtained from the Japanese Cancer Research Resources Bank (JCRB 0104.1), and maintained in RPMI 1640 medium supplemented with 10% fetal calf serum. To facilitate the growth and differentiation of basophils, the cells were cultured in the serum-free medium, COSMEDIUM-003 (COSMO-BIO), supplemented with 200 units/ml of recombinant human IL-3 (Genzyme) for 2–3 weeks [8].

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Abbreviations: PTP, protein-tyrosine phosphatase; PTK, protein-tyrosine kinase; FcεRI, high affinity IgE receptor(s); FACS, fluorescence-activated cell sorter; PCR, polymerase chain reaction; PSD-95, rat brain postsynaptic density protein; dlg, Drosophila lethal(1)discs-large-1 tumor suppressor protein; bp, base pair(s); kb, kilobase(s).

The nucleotide sequence data reported in this paper have been deposited in the DDBJ, EMBL and GenBank data bases under the accession numbers D21209, D21210 and D21211.

2.3. FACS sorting of basophils

Basophil-enriched cells were stained successively with human IgE, F(ab')₂ fragment of mouse anti-human IgE monoclonal antibody and FITC-conjugated anti-mouse IgG. After further staining with propidium iodide, the cells were analyzed on a FACStar^{PLUS} flow cytometer (Becton Dickinson). Fluorescence was measured at 530 (FL1) and 630 nm (FL2) for FITC and propidium iodide, respectively. Basophils exclusively expressing high-affinity IgE receptors in the culture cells, were sorted as the FL1 positive/FL2 negative fraction by FL1 and FL2 gating.

2.4. Oligonucleotide synthesis

Oligonucleotides were synthesized using a DNA synthesizer (CYCLONE, MilliGen). 5'-Fluorescein-labelled oligonucleotides were synthesized as above by means of fluorescein amidite and used as the primer for sequencing on an ALF DNA sequencer (Pharmacia). A pair of degenerate oligonucleotides, A and B, were designed from two highly conserved amino acid sequences within the PTP catalytic domain [9]. Oligonucleotides A (sense) and B (antisense) were based on the respective sequences of FWRM (I or V) W (E or Q) [5'-TT(C/T)TGGCGIATG(G/A)TITGG(C/G)A-3'] and HCSAG (V or I) G [5'-CC(G/A)A(C/T)CCNGCIGA(A/G)CA(A/G)TG-3'], where I is inosine and N is a mixture of A, C, G and T in oligonucleotide sequences.

2.5. PCR

2.5.1. Amplification of the PTP catalytic domain

Poly(A)⁺ RNA was isolated from 1.6×10^6 FACS-sorted basophils using a QuickPrep Micro mRNA Purification Kit, and used as the template to synthesize the first strand cDNA with an oligo(dT)15 primer and SUPERScript Reverse Transcriptase. Using the resultant first-strand cDNA as the template, the cDNA encoding the conserved PTP catalytic domain was amplified using AmpliTaq DNA polymerase in the presence of a pair of degenerate oligonucleotides, A and B, as the primer. After 25 cycles of amplification (94°C, 1 min; 50°C, 1 min; 72°C, 2 min) in a DNA Thermal Cycler (Cetus), the PCR product was cloned into the pCR II vector.

2.5.2. PCR walking in the 5' direction

The first-strand cDNA template was synthesized using the total RNA isolated from basophil-enriched KU812E cells and sequence-specific oligonucleotide primers. The 5' unknown regions were amplified by our PCR walking method [10].

2.5.3. Amplification of the 5'-end sequence of PTP-BAS

The extreme 5'-end sequence of PTP-BAS cDNA was amplified by a combination of a modified 5' RACE method and our two-step PCR [10]. Briefly, a poly(dC) sequence was added to the 3'-end of the first-strand cDNA described in Section 2.5.2 using terminal deoxynucleotidyl transferase following the 5' RACE System instructions. The second-strand cDNA was synthesized with an oligo(dG)15 primer tagged with a unique sequence. Using the resultant double-stranded cDNA as the template, two-step PCR was performed using two pairs of nested primers specific to unique tag and known BAS sequences as described [10].

2.6. Isolation of PTP-BAS cDNA

Total RNA was isolated from about 10^8 basophil-enriched KU812E cells by the acid guanidium/phenol/chloroform method [11], and poly(A)⁺ RNA was purified with a mRNA Purification Kit. From 3 µg of poly(A)⁺ RNA, a KU812E cell cDNA library was constructed using the SUPERScript Lambda System, the λZAP II/EcoRI Cloning Kit and Gigapack II Gold packaging extract. The resultant library contained 6.3×10^5 independent recombinants. The PCR fragment of a novel PTP (named NPTP) was radiolabeled using [α -³²P] dCTP and the Ready-To-Go DNA Labeling Kit. The KU812E cDNA library was screened with the radiolabeled NPTP probe at 60°C overnight in 5 × SSC, 5 × Denhardt's solution, 0.5% SDS containing salmon sperm DNA at 10 µg/ml. The filter was washed twice each with 2 × SSC, 0.1% SDS at room temperature for 10 min and with 1 × SSC, 0.1% SDS at 42°C for 15 min. Selected clones were plaque-purified and underwent *in vivo* excision.

2.7. Sequencing

The cloned cDNA fragment was sequenced on an ALF DNA sequencer (Pharmacia) using 5'-fluorescein-labelled oligonucleotide primers. The sequences cloned by PCR were finalized by sequencing at

least three independently amplified clones. PCR fragments amplified in Sections 2.5.2 and 2.5.3 were directly sequenced with a dsDNA Cycle Sequencing System. Sequencing was done on both strands.

2.8. Northern blot analysis

Expression of PTP-BAS in various human tissues was examined using Multiple Tissue Northern Blots (Human MTN and Human Fetal MTN Blots). The BAS cDNA fragment (BAS-1, Fig. 1) and a human G3PDH control probe were radiolabeled using the Ready-To-Go DNA Labeling Kit. The blot was hybridized at 42°C for 16 h in 5 × SSPE, 10 × Denhardt's solution, 50% formamide, 2% SDS containing salmon sperm DNA at 100 µg/ml. The blot was washed twice with 2 × SSC, 0.05% SDS at room temperature, twice with 1 × SSC, 0.1% SDS at 50°C, then twice with 0.1 × SSC, 0.1% SDS at 50°C for 20 min each before exposure to a Kodak XAR-film at -80°C with an intensifying screen.

3. Results

3.1. Identification of a novel PTP

To identify PTPs expressed in human basophils, sequences in the PTP catalytic domain were amplified by PCR using the first-strand cDNA from FACS-sorted basophils as the template. A pair of degenerate primers (A and B) corresponding to the sequences that are highly conserved but which flank a region of variable sequences was used. The predicted PCR product was cloned into the pCR II vector and a total of 33 clones was sequenced. Among these, 27 were known PTPs, including LCA [12,13], MEG [14], MEG2 [15] and iCTP [16]. The remaining six clones were identical and differed from the known PTPs. This PCR-amplified cDNA fragment was designated as NPTP (Figs. 1 and 2). 2.5×10^5 recombinants out of a cDNA library from basophil-enriched KU812E cells was screened with the radiolabeled NPTP probe. Two positive clones were isolated and sequenced (BAS-1 in Figs. 1 and 2). Four consensus polyadenylation signals (AATAAA) were found upstream from the poly (A) tail, but an in-frame stop codon preceding the initial methionine codon was not included, indicating that the coding region of BAS-1 extends further in the 5' direction.

To synthesize the first-strand cDNA containing the most 5' sequence of PTP-BAS, a sequence-specific oligonucleotide overlapping the 5'-end of BAS-1 was used as the primer. By using the resultant first-strand cDNA as the template, the 5' unknown sequence was specifically amplified by our PCR walking method [10]. Through six consecutive PCR walks, sequence of about 3 kilobases (kb) upstream from the 5'-end of BAS-1 was obtained. The 5'-end sequence of the PTP-BAS cDNA was amplified by our improved 5'RACE method (BAS-5 in Fig. 1) and overlapped with the sixth walking sequence. To verify the sequence obtained by the 5'PCR walking and 5'RACE methods, overlapping cDNA fragments (BAS-2, -3 and -4 in Fig. 1) were amplified using sequence-specific primers, cloned into pCR II vectors and sequenced. Two in-frame deletions were found in the BAS-

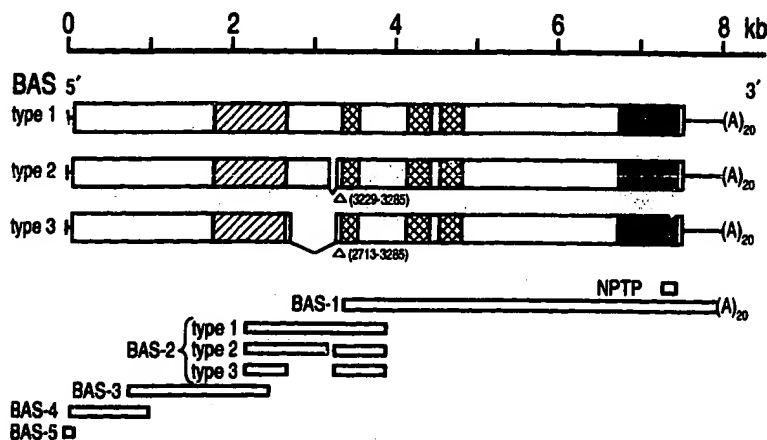


Fig. 1. The positions of the isolated BAS-1 and representative PCR fragments in the composite full-length PTP-BAS cDNA of three isoforms (types 1-3). The scale indicates nucleotide positions beginning from the first base of the cDNAs. Deleted segments in types 2 and 3 are shown by nucleotide numbers in type 1. The coding region of PTP-BAS isoforms is described by boxes: solid, catalytic domain; hatched, membrane-binding domain; cross-hatched, GLGF repeats. Thin bars indicate the sizes and relative locations of each cDNA fragment sequenced in this study.

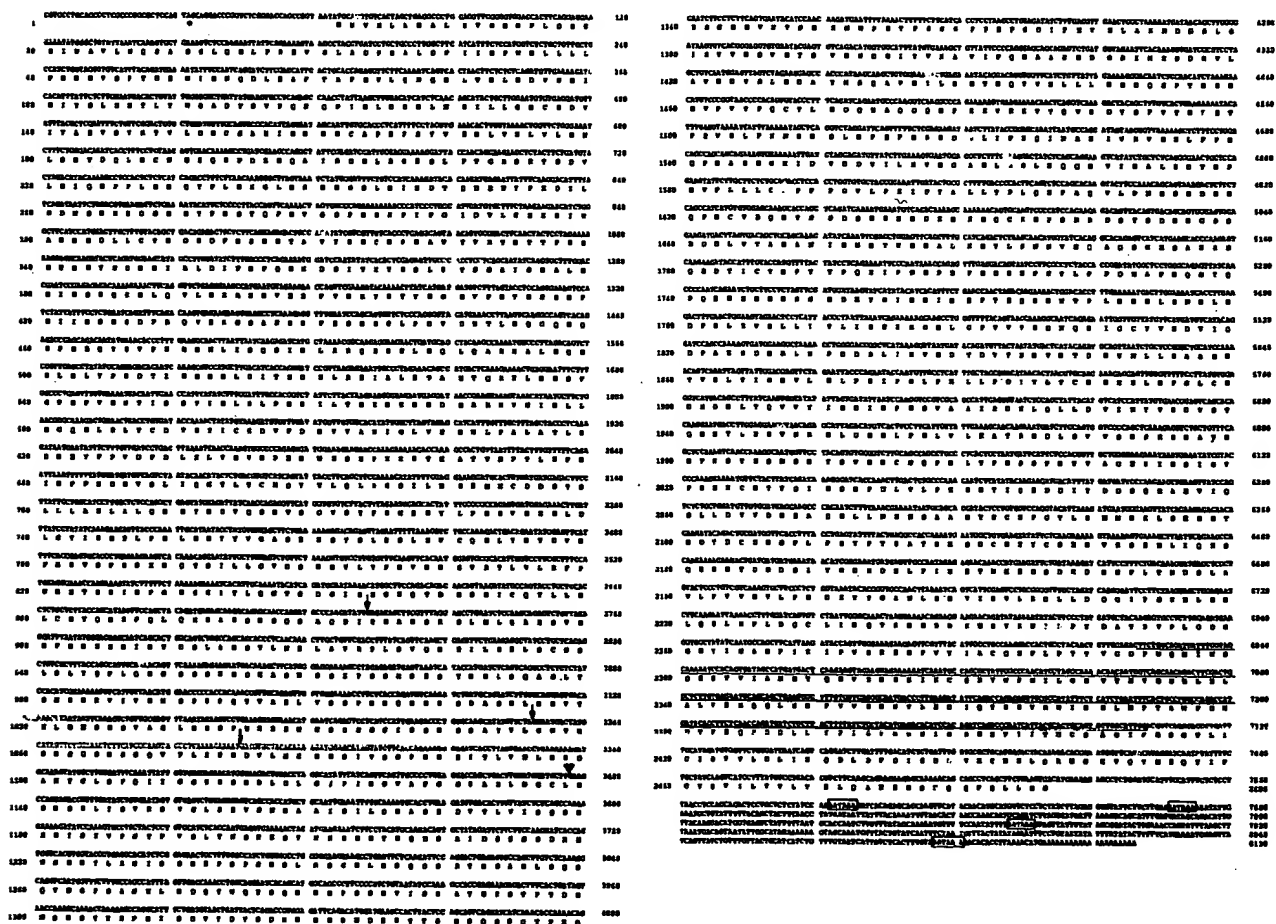


Fig. 2. Nucleotide and deduced amino acid sequence of PTP-BAS (type 1). The numbers on the right and left are respectively for the nucleotide and amino acid sequences. Positions of in-frame deletions for type 2 (nucleotides 3,229-3,285) and type 3 (nucleotides 2,713-3,285) are indicated by arrows. In-frame stop codons preceding and following the open reading frame are shown by asterisks. Consensus polyadenylation signals are boxed. The PCR product NPTP in the PTP catalytic domain is underlined. The 5'-end of the BAS-1 fragment is indicated by an inverted triangle.

BAS	GOTKENRRKNRYKNI LPYDTRVPLGDEGGYINASF I KIPVGKEEFVYIACQGLPTTVGD	2292
Consensus	... N NRY ... D R L ... Y NA ... QG ... T	
BAS	FWQMIWEQKSTV I AMMTQEVEGEK I KCQRYWPN I LGKTTMVSNNRLRLALVRMOQLKGFVVRA	2354
Consensus	... F W M W ... V ... E ... KC ... YWP	
BAS	MTLEDIQTREVRH I SHLNFTAWPDHDTSPDDLLTF I SYMRH I HRSGP I ITHCSAG I GR	2414
Consensus	... R ... WPD ... P ... G ... HCSAG GR	
BAS	SGTL I C I DVVLGL I SQDLDFDI SDLVRCMLQRHGMVQTEDQY I FCYQV I LVVLTALQA	2473
Consensus	... D ... R R ... Q Q	

Fig. 3. Sequence comparisons of the PTP catalytic domain in PTP-BAS and other known PTPs. The amino acid sequence of PTP-BAS (type 1) is numbered on the right. Consensus residues common to 12 representative PTPs (PTP1B [28], TCPTP [16], LAR [29], LCA [12,13], HPTP α , β , γ , δ , ϵ and ζ [9], DLAR [18], DPTP [18]) are shown [9]. Colons indicate identity with the consensus residues.

2 fragments, suggesting that PTP-BAS exists in three isoforms (types 1, 2 and 3).

The composite full-length nucleotide sequence designated PTP-BAS type 1 contains a consensus initiation codon (AATATGC) [17] at position 64 and is preceded by an in-frame stop codon (Fig. 2). The initiation codon is followed by a single open reading frame of 7,455 bp encoding 2,485 amino acids. Types 2 and 3 lack respectively, nucleotides 3,229–3,285 (amino acids 1,056–1,074) and nucleotides 2,713–3,285 (amino acids 884–1,074) in type 1 (Figs. 1 and 2). The open reading frames for types 2 and 3 are 7,398 bp (2,466 residues) and 6,882 bp (2,294 residues), respectively. All three isoforms of PTP-BAS contain a single PTP catalytic domain at their carboxyl termini (Figs. 1 and 2), where the cysteine (amino acid 2,408 for type 1) essential to catalysis [18] and 98% of 42 consensus residues common to 12 representative PTPs [9] are present (Fig. 3).

3.2. Sequence similarities

A homology search for the amino terminal region of

the PTP catalytic domain was conducted using the GenBank data base (release 78) with the FASTA program. Strong similarity (25–30% identity and 40–50% similarity) was revealed between amino acids 574–881 of PTP-BAS and the amino terminal sequences of cytoskeleton-associated proteins (protein 4.1, ezrin, radixin and moesin of human and other species) and two cytosolic PTPs (PTPH1 and PTP-MEG) (Figs. 4 and 6). Three GLGF repeats reported in the rat brain postsynaptic density protein (PSD-95) [19] as a homologue of the *Drosophila* lethal(1) discs-large-1 tumor suppressor protein (dls) [20] were found in the central part of PTP-BAS (amino acids 1,094–1,178, 1,368–1,452 and 1,501–1,588 for type 1) (Figs. 5 and 6). The repeats were 23–44% identical and 43–63% similar (Fig. 5).

3.3. Expression of PTP-BAS mRNA in the human tissues

Northern blots showed that PTP-BAS was expressed in all human tissues examined (Fig. 7). After normalization of the signal obtained with a G3PDH probe, the levels of PTP-BAS expression in adult human tissues are

BAS	VNIMLLNGQRLELTCDTKTICKDVDFMVAHIGLVEHHLFALATLKDNEYFFVDPDLKLTQVAPEGWKEPKKTKATV
Ezrin	VRVTTMDAE-LEFAIQPNTTGKQLFDQVVKTI GLREVWYFGLHYVDNKGFL-PTWLKLDKKYS-...AQEVKRN-PL
Radixin	VRVTTMDAE-LEFAIQPNTTGKQLFDQVVKTVGLREVWFFGLQYVDSKGY-...STWLKLNKKVT-...QGDVKKEN-PL
Moesin	VRVTTMDAE-LEFAIQPNTTGKQLFDQVVKTI GLREVWFFGLQYQDTKGF-...STWLKLNKKVT-...AQDVRKES-PL
BAS	NFTLFFRIKFFMDVSL-IQHTLTCHQYYLQLRKDI LEERMHCDDETSLLLASLALQAEYGDYQPEVHGVSYFRMEHYL
Ezrin	QFK-...FRAKFPEDVAEELIQDITQKLFFLOVKEGILSDEIYCPPEAVLLGSYAVQAKFGDYNKEVHKSGYLSSERLI
Radixin	QFK-...FRAKFPEDVSEELIQEITQRLFFLOVKEAILNDEIYCPPEAVLLASAVQAKYGDYNKEIHKPGYLANDRLI
Moesin	LFK-...FRAKFPEDVSEELIQDITQRLFFLOVKEGILNDDIYCPPEAVLLASAVQSKYGFNKEVHKSGYLAGDKLL
BAS	PARVMEKLDLSYIK-...EELPKLHNTYVGASEKETELEFLKVCQRLTEYGVHFRVHPEKKSQTGILLGVCSKGVLVFEV
Ezrin	PQRVMQHKLTROQWEDRIQVWHAHRGMLKDNAMLEYLKI AQDLEMYGINYFEIK-...NKKGTDLWLGVDALGLNIYEK
Radixin	PQRVLEQHLTKQWEEERIQWHEEHRGMLREDMMMEYLKI AQDLEMYGVNYFEIK-...NKKGTDLWLGVDALGLNIYEK
Moesin	PQRVLEQHLKNDQWEEERIQWHEEHRGMLREDAVLEYLKI AQDLEMYGVNYFSIK-...NKKGSELWLGVDALGLNIYEK
BAS	HNGVRTLVLRFPWRETKKISFSKKKITLQNTSDGIKH-GFQTDNSKICQYLLHLCSYQHKFQLOMRARQSNODAQ
Ezrin	DDKLTPKI-GFPWSEIRNISFNKKFV I KPIDKKAPDFVYAPRLRINKRI LQCMGNHLYMRRRKPDTIEVQQ
Radixin	DDKLTPKI-GFPWSEIRNISFNKKFV I KPIDKKAPDFVYAPRLRINKRI LQCMGNHLYMRRRKPDTIEVQQ
Moesin	NDRLTPKI-GFPWSEIRNISFNKKFV I KPIDKKAPDFVYAPRLRINKRI LQCMGNHLYMRRRKPDTIEVQQ

Fig. 4. Alignment of amino acid sequences 574–881 of PTP-BAS with membrane-binding domains (amino acids 7–304) of the representative human cytoskeleton-associated proteins: ezrin [30], radixin [31] and moesin [32]. Identical amino acids are indicated by asterisks. Conserved amino acid changes are indicated by dots.

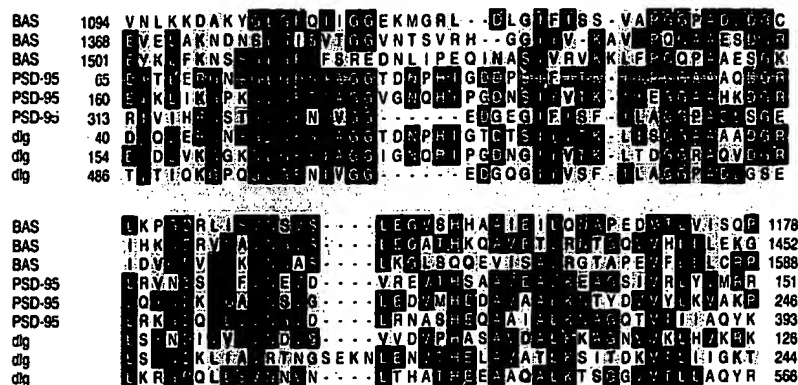


Fig. 5. Alignment of three GLGF repeats of PTP-BAS, PSD-95 [19] and *dlg* [20]. Amino acid sequences of each repeat are numbered. More than three identical amino acids at one position are highlighted by a black box. Conserved amino acid changes are shadowed.

as follows: the highest in the kidney; high in the lung; low in the placenta, brain, pancreas, heart and skeletal muscle; very low in the liver (Fig. 7A). Compared with the adult, the expression in human fetal (20–26 weeks) tissues was remarkably enhanced in the brain, but was similar in the kidney, lung, heart and liver (Fig. 7B).

4. Discussion

A novel PTP was identified from human basophils by PCR using degenerate primers deduced from conserved amino acid sequences in the PTP catalytic domain. A cDNA fragment of 4,663 bp (BAS-1 in Figs. 1 and 2) was cloned from a KU812E cDNA library. The 5' unknown region was amplified and sequenced by our modified 5' PCR walking and 5' RACE methods. The composite full-length cDNA contains consensus polyadenylation signals with a poly(A) tail and a consensus initiation sequence of Kozak [17] preceded by an in-frame stop codon (Fig. 2). The coding region contains two distinct in-frame deletions, suggesting that three isoforms (types 1–3) are expressed (Figs. 1 and 2). The estimated full-length cDNAs (8,139, 8,082 and 7,566 bp for types 1, 2 and 3, respectively) correspond roughly to the size of the

mRNA detected by Northern blotting (arrowed in Fig. 7), although the size differences were too small to be distinguished in the corresponding bands. A single PTP catalytic domain is present at the carboxyl termini of all isoforms, that contains all but one of 42 conserved residues in known PTPs [9] including the catalytically essential cysteine residue [18] at 2,408 for type 1 (Fig. 3). This novel PTP is named PTP-BAS (after basophils). A hydropathy plot of the deduced amino acid sequences showed no hydrophobic regions that resemble a signal sequence or transmembrane domain (data not shown), and thus all isoforms of PTP-BAS are likely to be cytoplasmic proteins. PTP-BAS is a cytosolic PTP of the largest molecule reported to date: 2,485 amino acid residues for type 1, 2,466 residues for type 2 and 2,294 residues for type 3 (Fig. 2). The existence in three isoforms may be of some functional importance.

A homology search for the amino terminal region of the catalytic domain revealed that all isoforms contain two distinct structural sequences (Fig. 1). Amino acids 571–881 of all the isoforms are highly similar to the amino terminal sequences of cytoskeleton-associated proteins: similar to the ezrin-radixin-moesin family [21] (27–29% identity and 50% similarity, Fig. 4) and somewhat less similar to protein 4.1 [22] (25% identity and

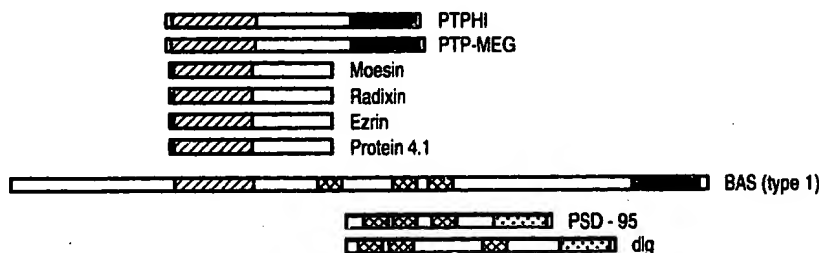


Fig. 6. A schematic diagram of structural domains of PTP-BAS (type 1) and its related proteins. PTP-BAS contains two distinct homologous domains: a membrane-binding domain (hatched bar) and three GLGF repeats (cross-hatched bar). Representative membrane-binding domains of human cytoskeleton-associated proteins (protein 4.1 [22], ezrin [30], radixin [31] and moesin [32]) and human cytosolic PTPs (PTP-MEG [14] and PTPHI [27]) are shown. Three GLGF repeats in cytoplasmic proteins (PSD-95 [19] and *dlg* [20]) are described. Other functional sequence shown in bars are PTP catalytic (solid) and guanylate kinase (stippled) domains. The length of each bar represents the actual length of the sequences.

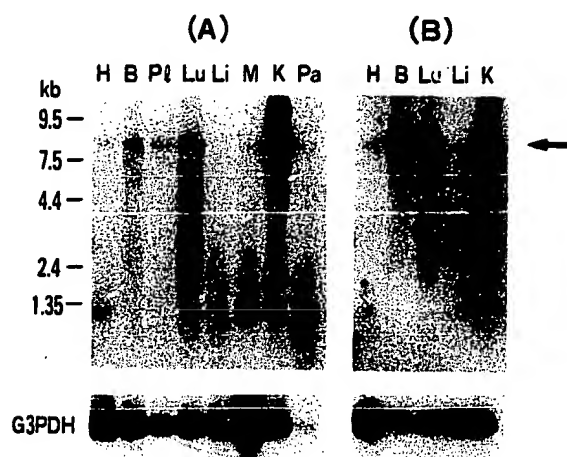


Fig. 7. Detection of PTP-BAS mRNA from various human adult (A) and fetal (B) tissues using multiple tissue Northern blots (CLONTECH). Each lane contains 2 μ g of poly(A)⁺ RNA. The blots were hybridized separately with the radiolabeled probes from BAS-1 and G3PDH cDNAs. PTP-BAS bands are marked by arrows. H, heart; B, brain; Pl, placenta; Lu, lung; Li, liver; M, skeletal muscle; K, kidney; Pa, pancreas. The numbers on the left are RNA sizes stated by the manufacturer.

41% similarity). Protein 4.1 binds to the cytoplasmic end of transmembrane proteins glycophorin [23] and band 3 [24] via the amino terminal domain [25]. Thus, the corresponding amino terminal region is called a membrane-binding domain [22]. Amino terminal domains of the ezrin-radixin-moesin family are homologous to the membrane-binding domain, so they are likely to bind to the plasma membrane [21,26]. The cytosolic PTPs containing the membrane-binding domain reported so far are PTP-MEG [14] and PTPH1 [27] (Fig. 6). PTP-BAS contains the other characteristic sequences: three 90 amino acid internal repeat sequences homologous to the GLGF repeats reported in guanylate kinase proteins: the rat brain PSD-95 protein [19] and the *Drosophila dlg* protein [20] (Figs. 5 and 6). These repeats are presumed to play a structural role in targeting the proteins to the submembranous cytoskeleton or a functional role of regulating the enzyme activity [19]. PTP-BAS is thus a novel cytosolic PTP, supposedly attached to the plasma membrane via the amino terminal membrane-binding domain and its substrate-specificity or PTP activity is regulated via the central GLGF repeats.

PTP-BAS initially cloned from human basophils was expressed in various human tissues, especially at the highest in the kidney and high in the lung. Interestingly, PTP-BAS was expressed at a remarkably high level in fetal, but weakly in the adult brain (Fig. 7). Furthermore, the PTP-BAS mRNA level increased with the growth and differentiation of human basophils cultured from cord blood mononuclear cells [10] (data not shown). Thus, PTP-BAS may play a role in intracellular signal transduction and may be relevant to cell growth and

differentiation. The functional significance of this novel PTP is now under investigation.

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